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Analysis of Resistance to Human Immunodeficiency Virus Type 1 Protease Inhibitors by Using Matched Bacterial Expression and Proviral Infection Vectors

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There are already reports, from clinical trials with human immunodeficiency virus type 1 protease inhibitors, of the emergence of drug-resistant mutants which have one or more point mutations in their protease genes. To examine roles of individual and multiple amino acid substitutions in terms of altered enzyme and virus drug sensitivities, we have produced matched vectors for bacterial expression and virus production. Both vectors accept the same restriction enzyme fragment, produced by PCR or PCR-mutagenesis of the protease gene, allowing parallel expression of mutant enzymes in *Escherichia coli* and in recombinant viruses. The utility of this vector system was demonstrated by using protease variants glycine to valine at amino acid 48 (G48V) and leucine to methionine at amino acid 90 (L90M) identified after passage of HIV-1 in the Roche phase II clinical trial protease inhibitor Ro 31-8959 (H. Jacobsen, K. Yasargil, D. L. Winslow, J. C. Craig, A. Krohn, I. B. Duncan, and J. Mous, Virology 206:527, 1995). G48V, L90M, and G48V/L90M exhibited successively less processing in vitro than the wild-type enzyme, and the purified enzymes were 220-, 20-, and 720-fold, respectively, less sensitive to Ro 31-8959. The reduced enzyme sensitivity correlated directly with the sensitivities of the matched recombinant viruses, in that individual mutations L90M and G48V conferred 2-fold and 4- to 6-fold increases in 50% inhibitory concentration, respectively, whereas G48V/L90M was 8 to 10 times less sensitive to Ro 31-8959. A proviral vector with the entire protease gene deleted was constructed for use as an in vivo recombination target for an overlapping protease PCR fragment, generating wild-type infectious virus. Finally, direct ligation of restriction fragments, generated from random PCR mutagenesis, into the proviral vector should provide a library of protease mutations that allow extremely rapid selection of highly resistant viral variants.

The protease (Pr) encoded by human immunodeficiency virus (HIV) is essential for virus growth, in that failure to cleave the Gag and Gag-Pol polyproteins to their mature forms results in the production of noninfectious particles (18, 32). For this reason, the HIV protease has proven to be an ideal target for chemotherapeutic intervention, and many inhibitors of the enzyme have shown potent antiviral activity (6, 25, 29, 38). Several of these protease inhibitors have subsequently entered phase I and phase II clinical trials for treatment of AIDS and AIDS-related complex, meeting with considerable success (1). However, in at least two instances, virus isolates that show decreased sensitivity to the inhibitor involved have emerged during therapy, and genotyping has shown in both cases that resistance involves single and multiple changes in the protease gene (8, 15). In addition, the changes seen in the clinic are largely those seen during resistance selection in cell culture (16, 26). Therefore, we and others have undertaken extensive studies in cell culture to monitor the emergence of protease drug resistance (3, 5, 7, 12, 16, 17, 23, 26, 35).

To analyze resistant variants isolated in cell culture and in the clinic, there is a need for a system that allows insertion of single and multiple mutations into an isogenic HIV provirus for virus production and sensitivity testing. Furthermore, high-level expression, purification and crystallization of HIV protease mutant enzymes would further aid the molecular and atomic determination of drug resistance. These requirements were addressed by us through development of matched vectors for virus and enzyme production on the basis of direct cloning of PCR fragments at unique enzyme restriction sites. Expression of the protease as a fusion protein in *Escherichia coli* and determination of precursor/product ratios in antiprotease Western blots (immunoblots) allows rapid analysis of mutant enzyme activity. Purified enzyme drug sensitivity correlated with virus drug sensitivity, using mutations identified from virus passage in cell culture and variants from clinical trials (15, 16). In addition, deletion of the protease gene from the proviral vector generated a target for recombination, in cell culture, with a homologous protease gene PCR fragment.

MATERIALS AND METHODS

PCR primers and mutagenesis. Fragments for direct cloning were generated by using a *Not*I 5′ primer (5′-CCTCAGAAG**GCGGCCGC**GATAGACAAGG-3′) and an *Asp*I-*Nhe*I 3′ primer, which also encodes a stop codon in the *Nhe*I site (5′-CC**GCTAGCCTGACACAGTC**TCAATAGGGCTAATGGG-3′). Recombinant virus assays (RVAs) (19) utilized primers RVP5′ (5′-GGGAAGATCTG GCCTTCCTACAAGGG-3′) and RVP3′ (5′-GGCAAATACTGGAGTATTGT ATGG-3′). PCR mutagenesis was performed by using the *Not*I or *Asp*I primer together with internal mutagenic primers as described by Landt et al. (20). The D25N 3′ primer sequence was 5′-CTGCTCCTGTAT**T**TAATCGAGC-3′, the G48V 3′ primer sequence was 5′-CTCCAATTCCC**A**CTATCATTTTTG-3′, and the L90M 5′ primer sequence was 5′-GGAAGAAATCTG**A**TGACTCAGATT GG-3′. The 5′ base of each primer corresponded to the third base in a codon triplet so that any *Taq* polymerase terminus errors were accommodated by anticodon wobble.

Plasmids (i) Preparation and sequencing. All plasmids were prepared from 5-ml cultures by using the Wizard Miniprep System (Promega) and were sequenced by using Sequenase version 2.0 (U.S. Biochemical).

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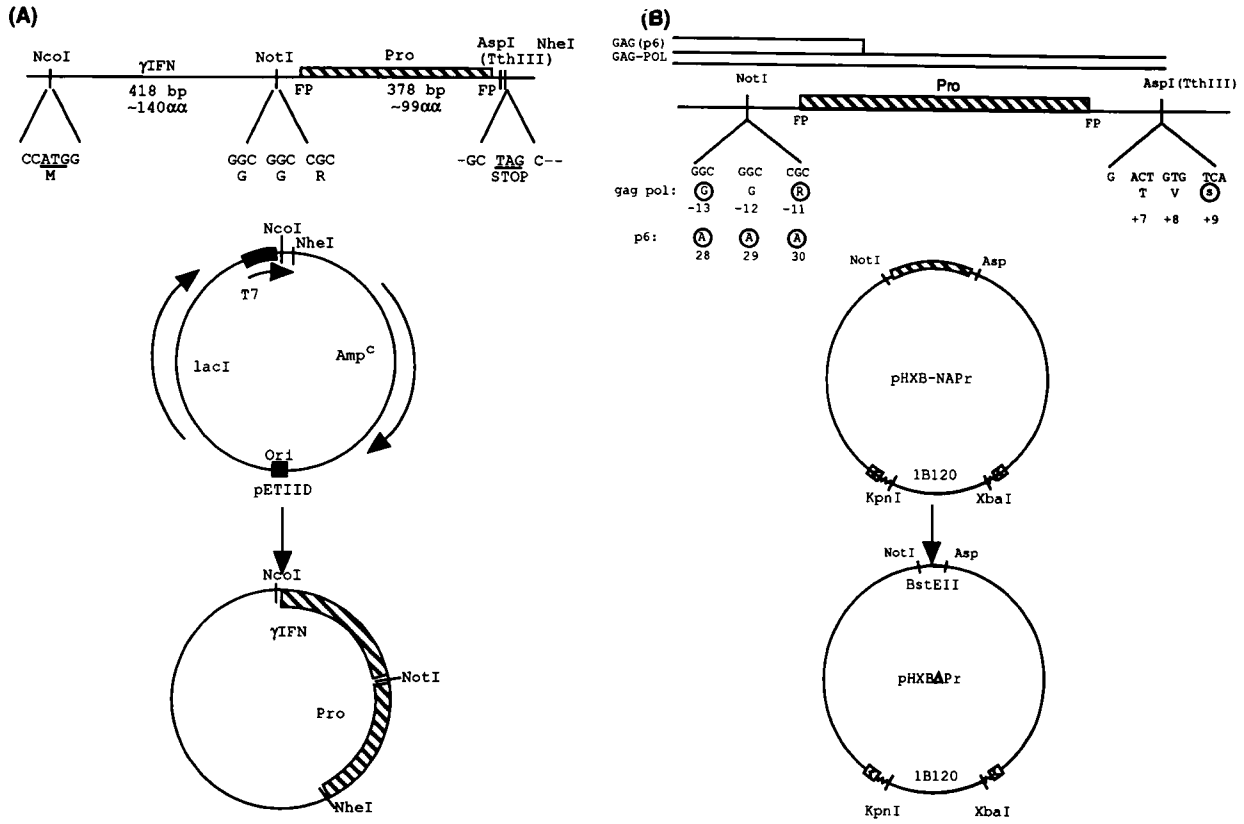

FIG. 1. Diagrams of the matched vectors for *E. coli* expression (A) and virus production (B). Full details of the plasmid structures are given in the text.

(ii) Bacterial vector. The gene for gamma interferon (IFN-γ) codons 1 to 139 was amplified from plasmid pIFG124A (22) by PCR using the IFN-γ 5′ (5′-G**C CATGG**GAGACCCATATGTACAAGAAGCAGAAAACC) and IFN-γ 3′ (5′-GCTCT**GCGGCCGC**CAAACAGCATCTGACTCC) primers (*Nco*I and *Not*I end sites), with restriction sites in bold type. The HIV protease gene was amplified from pIBI20HIV (9) by PCR with the *Not*I 5′ and *Asp*I 3′ primers (*Not*I and *Nhe*I end sites). The fragments were digested with *Not*I and ligated; correct-size IFN-γ–Pr ligation products were separated from IFN-γ and Pr dimers and then digested with *Nco*I and *Nhe*I. This fragment was ligated with *Nco*I- and *Nhe*I-digested pET11D (Novagen). The resultant plasmid, pT7-γIFN/H1PR, contains a single open reading frame (ORF) encoding an IFN-γ–HIV-1 protease fusion bridged by a glycine-glycine hinge (13), beginning at Gly-139 of IFN-γ (Fig. 1).

(iii) Proviral vector. The human genomic sequences flanking the HIV-1_{hxb2} provirus in pIBI20HIV were removed as follows. pIBI20HIV was digested with *Sma*I (cleavage in the pIBI20 polylinker) and *Hpa*I (cleavage in the 5′ flanking region [10]), and then blunt ends were joined to give a residual 5′ FLK sequence of 165 bp. This provirus was then digested with *Asp*I (cleavage in the 3′ flanking region), *Xba*I linkers were added, and after digestion with *Xba*I (cleaves at the junction of 3′ flanking sequences with the pIBI20 polylinker), the *Xba*I site was re-created with the residual 3′ FLK sequence of 293 bp. The 5′ and 3′ flanking sequence deletions removed about 6 kb of human sequences and greatly increased the stability of this proviral vector, pHXBΔFLK, particularly in *recA*$^+$ *E. coli* hosts. The Gag-Pr-reverse transcriptase (RT) *Kpn*I-*Sna*I DNA fragment was cloned into M13, site-directed mutagenesis was performed (T7-gen kit; U.S. Biochemical) to introduce unique *Not*I and *Asp*I sites 39 bp 5′ and 27 bp 3′, respectively, of the protease ORF, and then the entire fragment was cloned back into pHXBΔFLK, to give pHXB-NAPro. Double digestion of pHXB-NAPro with *Not*I and *Asp*I and insertion of a linker, recreating the *Not*I and *Asp*I sites and introducing a *Bst*EII site, generated plasmid pHXB-ΔPro. The sequence of the linker is

5′-GGCCGC**GGTGACC**GGACT
CG**CCACTGG**CCTGAC-5′

Bacterial expression. After sequencing of minipreps grown in *E. coli* JM109, plasmids with desired protease mutations were transformed into the *recA E. coli* strain HMS174(DE3)pLysS (Novagen); single colonies were grown to an optical density at 600 nm (OD_{600}) of 0.4 and then induced with 1 mM isopropylthiogalactopyranoside (IPTG). Expression was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, using antiprotease monoclonal antibody 7E1 (courtesy of Mary Graves, Roche [32]) and/or anti-IFN-γ sera (Serotec). Equal volumes of crude extracts were loaded in a range of 15 to 40 μg of protein per sample (Bio-Rad protein assay kit). Western blots were scanned on a Milligen Bioimager, and protease activity in crude soluble extracts was measured by using the Amersham HIV protease scintillation proximity assay. HIV protease was extracted with 50% acetic acid from the insoluble fraction of lysed *E. coli*, purified by Sephadex G-50, and refolded as described by Hui et al. (14). A typical enzyme assay mixture contained 100 mM morpholmepropanesulfonic acid (MES; pH 5.5), 400 mM NaCl, 0.5% dimethyl sulfoxide, 1.5 to 20 nM protease, 0 to 100 nM inhibitor Ro 31-8959 (supplied by I. Duncan, Roche [4]), and 10 μM fluorogenic substrate (36). With Ro 31-8959, the inhibition constant (K_i) values were comparable to the enzyme concentration in the assay (which violates a steady-state assumption). Therefore, a fit of equation 1 to the data was used to determine the enzyme concentration ([*E*]) and the inhibitor concentration protecting 50% of substrate from enzyme cleavage (IC_{50}), where *F* is the fraction of enzyme bound to the inhibitor and [*I*] is the total concentration of the inhibitor in the assay:

$$F = \frac{[I] + [E] + IC_{50} - \{([I] + [E] + IC_{50})2 - 4 \cdot [I] \cdot [E]\}^{1/2}}{2[E]} \quad (1)$$

K_i values were then determined from these IC_{50} values by using equation 2:

$$K_i = IC_{50}/\{1 + ([S]/K_m)\} \quad (2)$$

Detailed enzymological data will be published elsewhere (24), but statistical analysis was performed as follows. The constants defined by the nonlinear equations were estimated by using Sigma Plot (Jandel Scientific, Corte Madera, Calif.). Error estimates were from the error matrix generated during the fitting routine. Error estimates for values calculated from fitted values (for example, K_i values were calculated from IC_{50} values and K_m values) were determined by propagation-of-error analysis of Bevington (2).

Virological assays. Approximately 5 μg of proviral plasmid DNA, prepared from 5-ml cultures of *E. coli* JM109 and bearing desired mutations as determined by sequencing, was electroporated into T-cell lines (MT4, CEM-C8166, H9, and

H9-*tat* [provided by G. Pavlakis]) by the method described by Kellam and Larder (19). Cultures were monitored daily for appearance of cytopathic effect (CPE) and harvested when all cells were infected and/or lysed (usually day 7 or 8 postelectroporation). HIV p24 levels were determined in cleared cell culture supernatants by using a DuPont Merck kit, and virus titers were determined in MT4 cells by a 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) vital dye assay (27) or in HeLa CD4 cells by syncytium formation (34). Sensitivities of wild-type (wt) and mutant viruses to Ro 31-8959 were also determined by the MTT assay with MT4 cells. RVAs were performed as described previously (19), using 10 μg of *Bst*EII-linearized pHXB-ΔPro and RVP5′-RVP3′ PCR product. Total cell DNA was prepared from infected and uninfected cells by proteinase K digestion and phenol-chloroform-isoamyl alcohol extraction, and the protease region was amplified by using primers RVP5′ and RVP3′. The PCR products were doubly digested with *Not*I and *Asp*I or sequenced by using Sequenase version 2.0 (U.S. Biochemical), the *Not*I5′ and *Asp*I3′ primers, and the dimethyl sulfoxide method of Winship (37).

RESULTS AND DISCUSSION

Vector construction. Hostomsky et al. used the T7 expression system, employing M13-T7 polymerase phage and a dihydrofolate reductase-protease fusion protein with a polyglycine hinge, to express high levels of mature, self-cleaving HIV protease in *E. coli* (13). We surmised that autoprocessing of HIV protease from a precursor fusion during a short induction period in *E. coli* might give a crude but rapid measure of mutant enzyme activity by estimating precursor/product ratios. To overcome toxicity associated with protease expression prior to induction, we used a combination of the *lac* repressor and T7 lysozyme repression to negate basal expression but used chromosomal expression of T7 RNA polymerase to allow more uniformly induce precursor expression. As a fusion partner, we chose IFN-γ which we knew expressed at high levels in *E. coli* (22), and introduced a glycine-glycine linker (Fig. 1A).

Preparation of an infectious proviral vector had four requirements of the restriction sites to be used for cloning: (i) they should be unique within the vector, (ii) they should not introduce stop codons, (iii) they should not greatly alter amino acid sequences in the *gag* and *gag-pol* ORFs, and (iv) they should be very rare and thus infrequent within diverse HIV strains. On the 5′ side of the protease gene, a unique eight-cutter *Not*I site was selected. This enzyme site encoded two tandem glycine residues, ideal for the matched expression vector hinge, and introduced only two changes, one conservative, in the *gag-pol* ORF $A_{-13}G$ (numbering relative to amino acid [aa]1 of protease, P_{+1}) and $A_{-11}R$. In the *gag* ORF, three changes were introduced in a semiconserved region of the 52-aa p6 (Q28A, E29A, and P30A) (substitutions are indicated by the one-letter code and position for the original amino acid followed by the one-letter code for the substituted amino acid). Although the absence of p6 appears to affect virion release and *vpr* incorporation (11, 28), irrespective of the *vpu* background, truncations of p6 at aa 17 and 36, i.e., amino acids flanking the altered amino acids in our vector, resulted in only a moderate delay in virus growth kinetics of 1 to 2 days (11). The introduction of the unique nine-cutter *Asp*I site on the 3′ side of the protease ORF altered one amino acid in the RT N-terminal region, $P_{+9}S$, but this residue lies on the surface of RT and should not affect enzyme activity (31).

Vector plasmids are depicted in Fig. 1.

Protease expression in *E. coli*. Wild-type and D25N mutant protease expression was assessed both by bacterial viability and by SDS-PAGE and Western blotting. The alteration of aspartate 25, the catalytic center of the HIV protease, to the corresponding amide, asparagine, is known to completely inactivate the enzyme, and so true levels of precursor expression could be measured with this mutant.

Bacteria cultures were divided when the OD_{600} reached 0.4, half of the culture was induced with IPTG, and half was allowed to continue incubation in the absence of inducer. Noninduced cultures carrying the wt or D25N protease gene continued to grow to a maximum OD_{600} of 2, as did the induced culture carrying the D25N gene. Conversely, the induced wt culture maintained a static OD_{600} of 0.8, consistent with expression of the toxic HIV protease (Fig. 2A). The 2-h time samples were run on duplicate protein gels, one gel was stained with Coomassie blue, and the contents of the other gel were transferred onto nitrocellulose and probed with a monoclonal antibody specific for HIV-1 protease (Fig. 2B). The D25N fusion protein is expressed at high levels as an abundant 27-kDa polypeptide (lane 3). This protein is absent from both uninduced and wt-induced protease samples. The Western blot confirmed that this 27-kDa protein was the IFN-γ–protease fusion. A small quantity of fusion protein was detected in the wt-induced sample, but most of the protease reactivity is found in the fully processed form at about 11 kDa (lane 5). The minor species immediately below the 27-kDa band and above the 11-kDa band probably represent partially processed intermediates with only C-terminal or N-terminal cleavages, respectively. The relative abundance of the minor bands suggests that the N-terminal cleavage is more efficient than the C-terminal cleavage, consistent with previous reports (21).

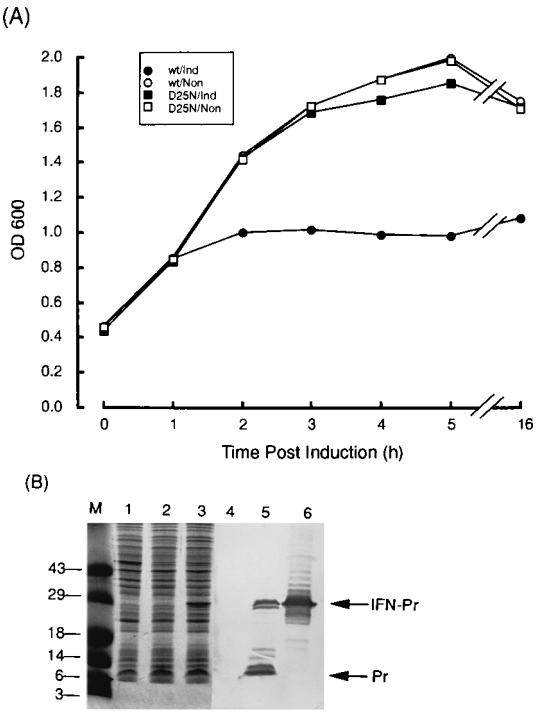

FIG. 2. Expression of wt and D25N proteases in *E. coli*. (A) Bacterial viability with (Ind) and without (Non) IPTG induction was monitored by reading the A_{600}. (B) Whole cell extracts were prepared at the times postinduction in panel A, and gels were run. The 2-h matched samples are shown as both Coomassie blue-stained gels and antiprotease Western blots. Lanes: M, size markers (indicated in kilodaltons); 1 and 4, wt not induced; 2 and 5, wt induced; 3 and 6, D25N induced.

Protease expression from proviruses. Proviral constructs pHXBΔFLK (wt protease), pHXB-NAPr (NAPr denotes the wt protease gene flanked by *Not*I and *Asp*I sites), and pHXB-D25N (mutated protease gene D25N with *Not*I and *Asp*I sites) were electroporated in parallel into four T-cell lines, CEM-C8166, H9, H9-*tat*, and MT4, and virus yield was determined by HeLa CD4 plaque assay 10 days after electroporation. In all

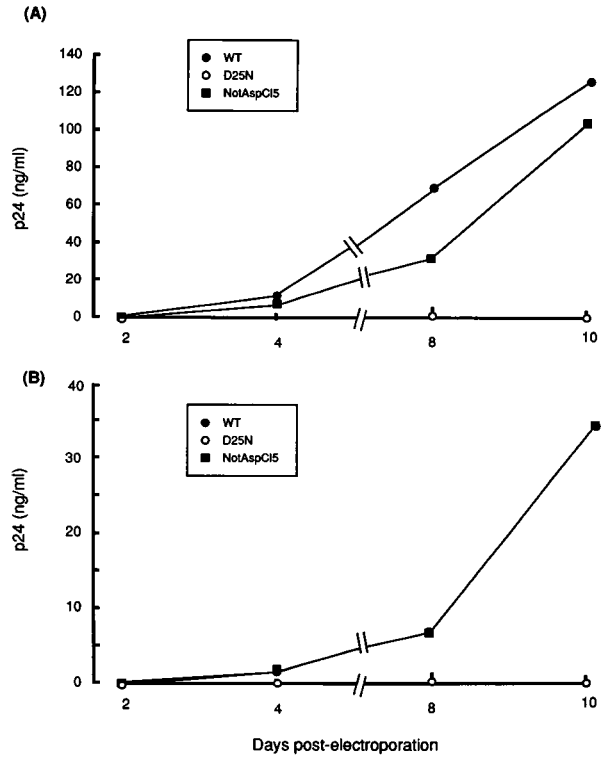

FIG. 3. p24^{gag} levels monitored at days 2, 4, 8, and 10 after electroporation of MT4 (A) or CEM-C8166 (B) cells, with the wt proviral clone, pHXBΔFLK, the modified clone pHXB-NAPr, containing *Not*I and *Asp*I restriction sites, and mutagenized pHXB-NAPr with a D25N alteration in the protease gene.

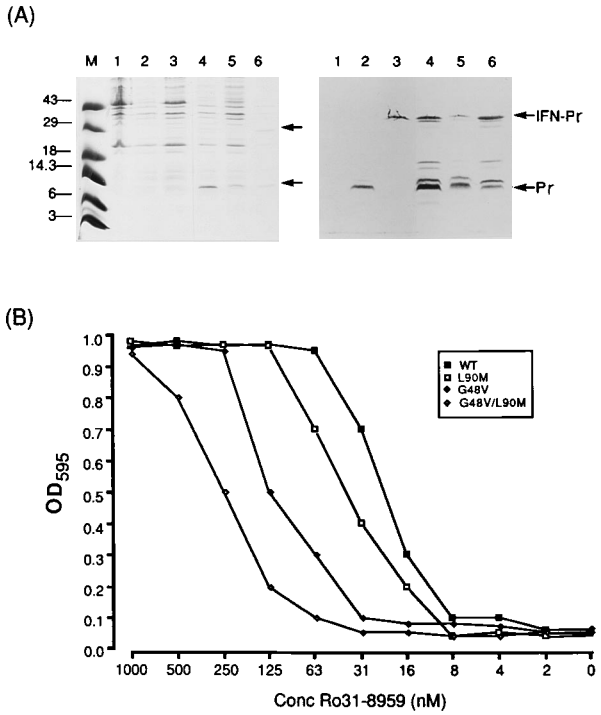

FIG. 4. Effects of G48V and L90M mutations on enzyme autocatalysis (A) and virus sensitivity to Ro 31-8959 (B). (A) Crude samples were prepared 2 h after IPTG induction, and equal volumes were electrophoresed. A Coomassie blue-stained gel and antiprotease Western blot are shown. Lanes: M, sidemarkers (indicated in kilodaltons); 1, uninduced wt; 2, induced wt; 3, D25N; 4, G48V; 5, L90M; 6, G48V/L90M. Fusion protein and mature product are shown. (B) Virus sensitivity was determined by measuring the OD_{595} of the solubilized tetrazolium vital dye MTT as described previously (27).

four cell lines, D25N proviruses produced no detectable virus. In CEM-C8166, H9, and H9-*tat* cells, the titers from pHXBΔ FLK and pHXB-NAPr were very similar, at 1×10^3 to 2×10^3 PFU/ml. However, in MT4 cells, the titer of pHXBΔFLK was 9×10^3 PFU/ml, whereas pHXB-NAPr gave a titer of 5×10^3 PFU/ml; in other experiments, the two proviral plasmids have given very similar titers. In the same experiment, supernatants were taken at days 2, 4, 8, and 10, and p24 levels were measured. In CEM-C8166 cells, as in H9 and H9-*tat* cells, p24 levels were identical for both wt protease constructs (Fig. 3B) but negligible for the D25N construct and reached 35 ng/ml at day 10. The levels of p24 were much higher in MT4 cells, reaching greater than 100 ng/ml at day 10 for both pHXBΔ FLK and pHXB-NAPr and being again negligible for the D25N provirus, but pHXB-NAPr-electroporated cells produced lower p24 levels at all time points in this assay (Fig. 3A), reflecting the day 10 HeLa CD4 titers. Although such a lag in p24 levels could be due to changes in p6 (11, 28), we believe that the lag is more likely due to electroporation efficiency, since subsequent experiments gave identical HeLa CD4 and p24 titers for pHXB-NAPr and pHXBΔFLK in MT4 cells (2a). MT4 consistently gave higher yields of virus from electroporations, and so in all subsequent experiments, virus stocks were produced in MT4 cells.

Effects of G48V and L90M mutations on enzyme activity and sensitivity to Ro 31-8959. To test the utility of the matched vectors, we tested the effects of single and double mutations, selected by cell culture passage of virus in the presence of Ro 31-8959 (16), on the activity of the protease enzyme and virus drug sensitivity. G48V is the first mutation observed in the virus population in cell culture, being joined in later passages by the L90M mutation (16, 35).

We prepared plasmids expressing G48V protease, L90M protease, and G48V/L90M protease. Expression of mutant proteases G48V and G48V/L90M appeared to have a smaller effect on bacterial viability, as monitored by OD_{600}, than the wt enzyme, whereas L90M had an effect equivalent to that of the wt enzyme. In Coomassie blue-stained gels, the relative level of precursor proteins appeared to be higher than for the wt enzyme for all three mutants (Fig. 4A). This finding suggested slower processing during the 2-h induction period. Some quantitative measure of relative processing activity was obtained by scanning duplicate gels that were used for antiprotease Western blots (Fig. 4A, lanes 1 to 6). These blots showed that wt protease processed about 100% precursor to product (and D25N processed 0%), G48V processed 76%, L90M processed 55%, and G48V/L90M processed only 31%. Thus, although the mutations may confer drug resistance, the enzymes appear to autocatalytically be less active.

When the purified enzymes were tested for sensitivity to Ro 31-8959, the K_i for L90M, G48V, and G48V/L90M mutants against the inhibitor were 20-, 220-, and 720-fold higher, respectively, than that of the wt enzyme (Table 1). Although the rank order of mutant virus resistance correlates with the increase in K_i for the mutant protease, the magnitude of the viral resistance increase is considerably smaller than the magnitude of the K_i increase. For example, the G48V virus is 6-fold resistant, whereas the G48V protease has a 220-fold-increased K_i value (Table 1). Therefore, this mutation has a 35-fold-larger affect on the enzyme binding than it does on the virus

TABLE 1. K_i values for the three mutant enzymes and wt enzyme and IC_{50} values for the same protease mutants as viruses

Construct	Enzyme		Virus	
	Mean K_i (nM) ± SD	Fold increase[a]	IC_{50} (nM)	Fold increase
wt	0.018 ± 0.003	1	26	1
L90M	0.36 ± 0.06	20	49	1.9
G48V	4 ± 1	222	142	5.5
G48V/L90M	13 ± 2	722	240	9.2

[a] Determined by dividing the mutant K_i or IC_{50} value by the corresponding value for wt enzyme or virus.

resistance. There are many possible explanations for the differential effects of mutations on enzyme compared with virus. The most obvious is that cell-based assays reflect many parameters that affect the efficacy of a given compound, even relative to the wt virus. The enzyme assay measures inhibition of cleavage of a single peptide, whereas inhibition of HIV replication is dependent on cleavage at eight or more polypeptide sites. Therefore, a defined reduction of enzyme activity may not necessarily result in the same reduction of viral replication, although Kaplan et al. (18) showed that viral replication was severely hindered by inhibition of only a small fraction of *gag/pol* processing. Detailed enzymatic measurements will be described elsewhere (24); these studies include assays with peptides corresponding to the p6/Pr and Pr/RT sites so that relative autocatalysis efficiencies can be compared with k_{cat} and K_m values.

Effects of G48V and L90M mutations on virus sensitivity to Ro 31-8959. The corresponding mutant viruses were prepared by electroporation of MT4 cells with defined proviral clones prepared in the pHXB-NAPro vector. MT4 genomic DNA prepared from day 8 cultures was used as a target to PCR amplify the protease region for sequencing, which confirmed that the desired mutations were maintained (data not shown). The day 8 culture cell-free supernatants were used to determine HeLa CD4 titers, which were all approximately 5×10^3 PFU/ml, and a uniform multiplicity of infection (0.001) was used for drug sensitivity assays. IC_{50} values were 26 nM for the wt virus, 49 nM for L90M, 142 nM for G48V, and 240 nM for G48V/L90M (Fig. 4). Repeat assays gave similar relative increases in IC_{50} values; with $2 \times IC_{50}$ for L90M, $6 \times IC_{50}$ for G48V, and $10 \times IC_{50}$ for G48V/L90M. These values agrees very closely with the fold increases in sensitivity observed by Jacobsen et al. (16) for L90M ($3K_i$ for the corresponding purified enzymes (Table 1). Genetically pure virus stocks are stable over at least two passages in cell culture in the absence of drug, although the single L90M mutation can be outgrown when mixed with wild-type virus (2a).

Deletion of the protease gene produces a target vector for in vivo recombination. Kellam and Larder (19) described an RVA for generating isogenic viruses with mutations in the RT gene. To test whether such a system would work for protease, we deleted the protease gene from pHXB-NAPr and replaced it with a linker containing a unique *Bst*EII site. This plasmid, pHXBΔPr, was linearized with *Bst*EII and electroporated into MT4 cells with and without a PCR product, generated with primers RVP5′ and RVP3′, which had 5′ and 3′ overlaps of 125 and 141 bp, respectively. CPE was apparent at day 12 in cultures in which both linearized plasmid and PCR product were mixed, but no CPE appeared in cultures electroporated with either plasmid or PCR product alone. In a parallel culture, CPE from pHXB-NAPr appeared at day 8. Genomic

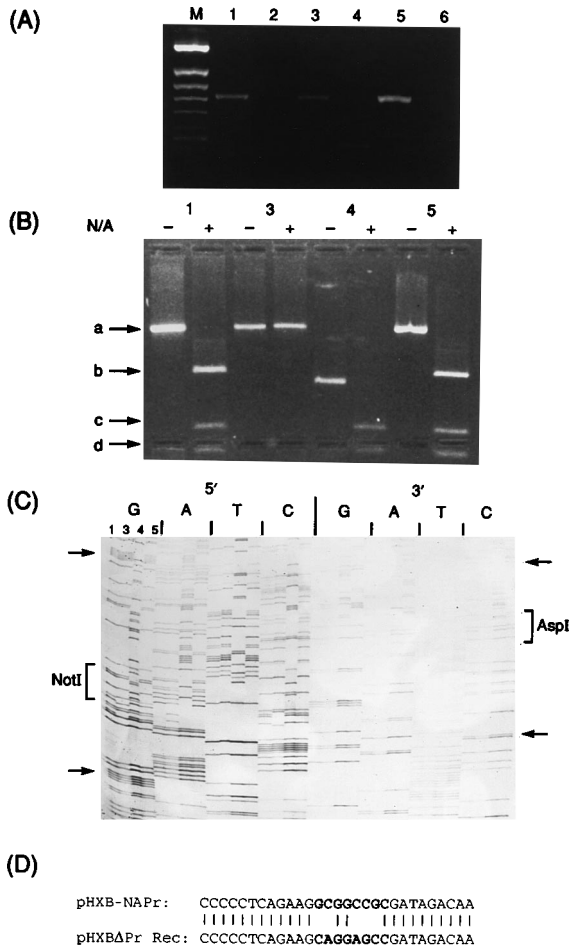

FIG. 5. Comparison of genomic proviral sequences after electroporation of MT4 cells with pHXB-NAPr (wt) (lane 1), PCR product only (lane 2), pHXBΔPr plus PCR product (lane 3), and pHXBΔPr only (lane 4). Lanes 5 and 6 are positive and negative PCR controls; lane M contains size markers. Genomic DNA from electroporated cells was subjected to PCR with primers RVP5′ and RVP3′ (A), and the PCR products were either not digested (−) or digested with *Not*I and *Asp*I (N/A) to resolve full-length product a from internal fragment b and flanking fragments c and d. The same PCR products were sequenced to ensure that in vivo recombinant was fully wt in the protease region (C), where the locations of the *Not*I and *Asp*I sites are marked; arrows indicate colinearity of the NAPr and wt Pr region. (D) Sequence read from the gel, confirming production of fully wt virus by in vivo recombination. Boldface letters indicate differences between NAPr and wt Pr.

DNA from these cultures was used to PCR amplify the protease region, with primers RVP5′ and RVP3′, and digested with *Not*I and *Asp*I (Fig. 5B) or sequenced (Fig. 5C). The RVA-derived PCR product has lost both restriction sites, and the sequence across the region was the wt sequence, including the stretches previously occupied by restriction sites.

The protease deletion in pHXBΔPr maintains the remainder of the *pol* gene in frame with the +1 frameshift, which may permit *trans* complementation by a protease-expressing plasmid. This possibility is untested as yet. The p6 ORF in this plasmid is truncated at aa 38 and so should be equivalent in function to the Y36/s p6 described by Gottlinger et al. (11).

Conclusions. We believe that the matched-vector system has several valuable features. First, the use of PCR products and the utility of the PCR mutagenesis technique make it both quick and simple to generate single and multiple point mutations for protein expression and virus production. Second, the

levels of expression with the bacterial vector suggest that it should be feasible to purify most mutant proteases and so relate enzymological parameters to virus drug sensitivities. Third, in terms of mutant virus production, the preparation of genetically pure provirus in *E. coli*, rather than in cell culture from homologous recombination, means that even viruses with quite severe growth disadvantages can be prepared. Finally, we have used the PCR random mutagenesis method of Siderovski et al. (30) to generate a library of mutant protease *Not*I-*Asp*I fragments, which were inserted into the proviral vector. This proviral library was amplified without cloning in *E. coli* JM109 and when electroporated into T cells should aid very rapid identification of mutants arising during selection with any protease inhibitor.

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